Ion Chemistry of Deprotonated Phenylthiocarbamyl–Phenylalanine

Xiaolei Li and Edmond de Hoffmann
Université Catholique de Louvain, Service de Spectrométrie de Masse, Louvain-la-Neuve, Belgium

The fragmentation of an $[M-H]^-$ anion of phenylthiocarbamyl (PTC)–phenylalanine (Phe) and of a deuterium labeled analog using electrospray ionization (ESI) in a negative ion mode was studied. Product ion experiments show that deprotonated PTC–Phe fragments mainly through three different pathways. Further tandem mass spectrometry experiments show that these three pathways actually correspond to the three different deprotonation sites in the molecule. These three ionization sites lead to the losses of, respectively, H_2S and (H_2S + CO_2), $C_6H_5N{=}C{=}S$, and $C_6H_5NH_2$ from $[M-H]^-$ in the first steps. They yield anions stabilized by resonance. The fragment produced by the loss of $C_6H_5NH_2$ is relatively weak and originates from a less acidic site. By selecting these fragment anions successively as precursors from the ion source, detailed information on the fragmentation mechanism is obtained. The benzyl-type anion plays a stabilizing role through conjugation. The fragmentation process also involves some uncommon neutral losses. For example, losses of HCN involve reactions that may occur through four- or five-membered cyclic transition states and cyanide ion–molecule complexes. Similar losses of HCN may occur through both the carboxylate anion and one of its tautomeric forms. Deuterium labeling studies support all the mechanistic proposals. The fragments obtained following the loss of the derivatization reagent ($C_6H_5N{=}C{=}S$) show that it is the anion of the deprotonated free amino acid. Preliminary results have shown that this also occurs for other PTC amino acids. (J Am Soc Mass Spectrom 1997, 8, 1078–1084) © 1997 American Society for Mass Spectrometry

A number of publications have appeared dealing with mass spectra of various types of derivatives from free amino acids in order to confirm their identification after a chromatographic separation. Most of them were conducted in a positive ion mode with different ionization techniques [1].

Recently some methods have been developed to analyze amino acid mixtures from biological samples, using tandem mass spectrometry. These methods are based on the classical formation of *n*-butyl esters of the amino acids. These derivatives are analyzed by tandem mass spectrometry of positive ions from either liquid secondary ion mass spectrometry or, more recently, electrospray ionization (ESI) [2, 3]. The scan mode used to selectively detect these amino acid derivatives relies on several neutral loss scans. For example, the neutral loss of 102 u detects many amino acid butyl esters, while the loss of 119 Da is typical of basic amino acids; other scans allow detection of other amino acids classes [4–6].

Phenylthiocarbamyl (PTC) amino acids or phenylthiohydantoin (PTH) amino acids are frequently used derivatives. With the phenylisothiocyanate (PITC), the reaction strongly depends on the experimental conditions used. Results described in the literature for hydantoin derivatives show that the main fragments occur from the derivative reagent portion, and do not allow to distinguish the amino acids from each other by the fragmentation pattern [1]. Mass spectrometry has not become an important analytical method for the analysis of the classical PTH and PTC amino acid derivatives, probably owing to availability of powerful techniques of high-performance liquid chromatography (HPLC) [7–10]. Mass spectrometry has been used only to confirm the results of various chromatographic techniques.

Formation of the phenylisothiocarbamyl (PTC) amino acid derivatives is shown in Scheme I.

We have observed that these compounds under electrospray ionization produce abundant negative ions of the deprotonated molecule. Their tandem mass spectrometry fragmentation allows to observe cleavages that are typical of each amino acid. We report here the reactions observed from PTC-phenylalanine, which was used to obtain details on the fragmentation mechanism.

Experimental

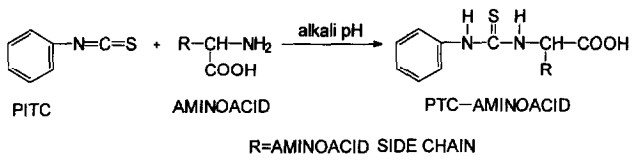
Materials

All solvents used were high purity grade (HPLC grade). Pyridine, heptane, ethyl acetate, and acetonitrile were obtained from Aldrich (Milwaukee, WI). Water used for

Address reprint requests to Edmond de Hoffmann, Spectrométrie de masse, Place Pasteur, 1, 1348 Louvain-la-Neuve, Belgium. E-mail: dehoffmann@cico.ucl.ac.be

1044-0305/97/$17.00
PII S1044-0305(97)00154-2

Received April 14, 1997
Revised June 13, 1997
Accepted June 16, 1997



Scheme I. Formation of PTC amino acids derivatives under basic condition.

the preparation of these standard solutions was deionized (Milli-Q water purification system, Millipore, Bedford, MA). The reagents, phenylisothiocyanate (supplied in 1-mL flame sealed amber ampules), a standard mixture of amino acids (2.5 μmol/mL each in 0.1 N HCl), and a kit of individual amino acid standards, were purchased from Sigma Chemical Co. (St. Louis, MO). Stable isotope standard phenylalanine-ring-$^2H_5$ was purchased from Cambridge Isotope Laboratories [Advanced Research in Chemistry (A.R.C.)].

Derivatization

Phenylthiocarbamylation of the amino groups of amino acids under the conditions described herein proceeds readily in a matter of a few minutes at room temperature. We used the method described by Chait et al. [11] with some modifications: 5 μL of phenylalanine (1 nmol/μL) and 5 μL of [$^2H_5$]-phenylalanine (1 nmol/μL) were added into a 1.5-mL polypropylene microcentrifuge tube; 50 μL of pyridine/water (1:1 v/v) (to maintain the optimum pH = 10) and 5 μL of the coupling reagent containing phenylisothiocyanate/pyridine (1:4 v/v) were added to the reaction vial. The vial was capped, and vortexed at room temperature for about 10 min. The resulting mixtures were extracted by adding 100 μL of heptane/ethyl acetate (10:1 v/v) in order to remove the excess coupling reagent. After gentle vortexing, the phases were separated by centrifugation and the upper phase was discarded. This washing procedure was repeated once, followed by washing twice with heptane/ethyl acetate (2:1 v/v). The remaining solution containing the amino acid products was dried by nitrogen, and was reconstituted in 100 μL of acetonitrile/water (3:1 v/v). By diluting this solution before tandem mass spectrometry analysis, the sample concentration is adjusted in the range of subpicomole per microliter. The sample was filtered through a 0.2-μm membrane filter (Microgon, Laguna Hills, CA) prior to analysis by ESI.

Mass Spectrometry

ESI-MS/MS was carried out on a Finnigan MAT TSQ-7000 triple quadrupole mass spectrometer equipped with an electrospray ion source. Xenon was used as collision gas at a pressure of 0.8 m torr. Tuning was optimized by using a standard amino acid solution converted to PTC derivatives. The concentration of each free amino acid was 2.5 μmol/mL with the exception of the cystine, whose concentration was 1.25 μmol/mL in the solution. The same procedure for derivatization was followed as described above. The settings for the ion source were optimized first to achieve maximum intensity of the $[M-H]^-$ ion with unit mass resolution in the first quadrupole. The optimum ESI-MS/MS conditions were established and the collision potential was set to 12-V offset voltage for all tandem mass spectrometry experiments. The second analyzer (Q3) was also set to unit mass resolution for the product ion. The capillary temperature was maintained at 240°C. The electrospray voltage was 4400 V and a flow rate of 3.0 μL/min was used. This low spray voltage reduces the ionization of the solvent molecules, thus reducing the background. Neither sheath gas nor auxiliary gas was used. Signal processing was done according the procedure described by Chace et al. [6]. Thirty-four consecutive scans of 1 s each were accumulated into a single raw spectrum, which was then processed by five-point smoothing. The result of this was a profile mass spectrum with much better signal-to-noise ratio and reproducibility. However, the appearance of the peaks is somewhat broadened by the smoothing process. Reproducibility was checked by reloading a fresh sample and comparing relative abundance of the ions. Typical variation was less than 5%.

Results and Discussion

To be in a concentration range realistic for biological samples, the derivatization process was performed on 1-nmol total amount of amino acid. After workup, the final solution was 100 μL, which corresponds to 10 pmol/μL if there is no loss, which is obviously not true. This solution was injected into the electrospray source at a rate of 3 μL/min. Under these conditions, the intensity of the peaks in the molecular ion region is quite low, as compared to the lower mass background peaks, however, the peaks give a good signal-to-noise ratio, allowing the observation of the isotopic peaks. The PTC amino acid derivatives obtained in our experimental conditions are well suited for the analysis by negative-ion ESI-MS since they carry a carboxylic acid function that is easy to ionize, yielding a negative charge. Accordingly, no additives are needed in the mobile phase. Very good quality tandem mass spectra are obtained.

The ESI-MS/MS product ion spectrum from the PTC-Phe $[M-H]^-$ anion is presented in Figure 1.

Three major fragmentation pathways can be described from Figure 1, which are represented by three different styles of labels for the ions. Fragments result from the three initial $[M-H]^-$ anions at the same 299 formed by the different deprotonations of the three acidic positions in the PTC–Phe, as will be shown by deuteration later. From these pathways, several main fragments are observed. They result from the losses of H_2S (34 u), [H_2S + CO_2] (78 u), $C_6H_5NH_2$ (93 Da), and

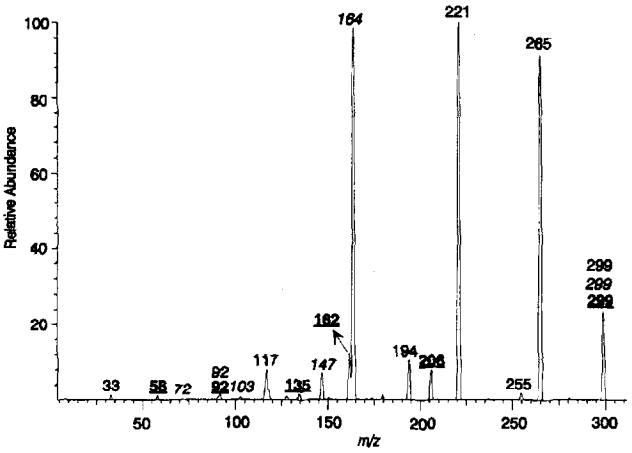


Figure 1. ESI-MS/MS product ion spectrum from PTC-Phe $[M-H]^-$ anion. There are three major fragmentation pathways represented by three different text styles.

$C_6H_5N{=}C{=}S$ (135 Da), yielding the peaks observed at 265, 221, 206, and 164, respectively. The fragment at m/z 206 is weaker and originates from a less acidic site.

The ESI-MS/MS spectrum of the labeled (phenylalanine-ring-$^2H_5$) PTC-Phe $[M-H]^-$ ion is given in Figure 2. It shows that the peaks at m/z 299, 265, 255, 221, 206, 194, 164, 162, 147, 135, and 103 in the spectrum of unlabeled PTC-Phe (Figure 1) were shifted by five mass units higher in the spectrum of the labeled $^2H_5$ compound (Figure 2). These shifted peaks indicate ions containing the phenyl group of the phenylalanine moiety. There are also common peaks at m/z 117, 92, 72, 58, and 33 that can be observed both in Figures 1 and 2. The common masses mean that the phenyl group from the phenylalanine moiety is not present in those fragments.

A $^2H_4$ labeled PTC-[$^2H_4$] Phe analog is present as an about 2% impurity of the $^2H_5$ ring deuterated compound. Its fragmentation spectrum ($^2H_4$, m/z 303) shows exactly the same fragmentation pathways either by shifting four mass units higher for the ions containing the phenyl group of the phenylalanine moiety or by displaying the common ions (spectrum not shown). The method thus proved to be very sensitive and yielded reproducible fragmentation using such low sample concentrations.

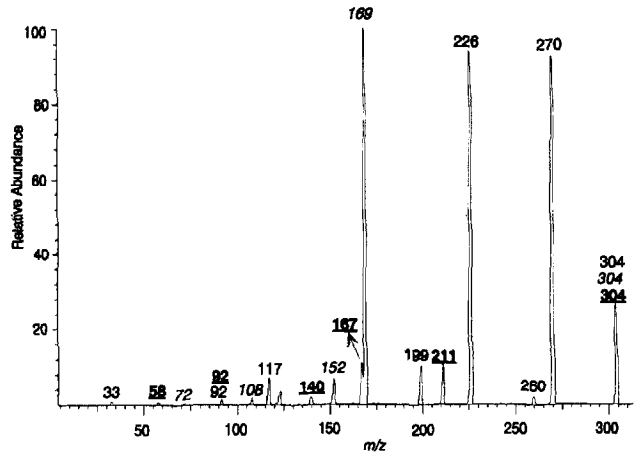
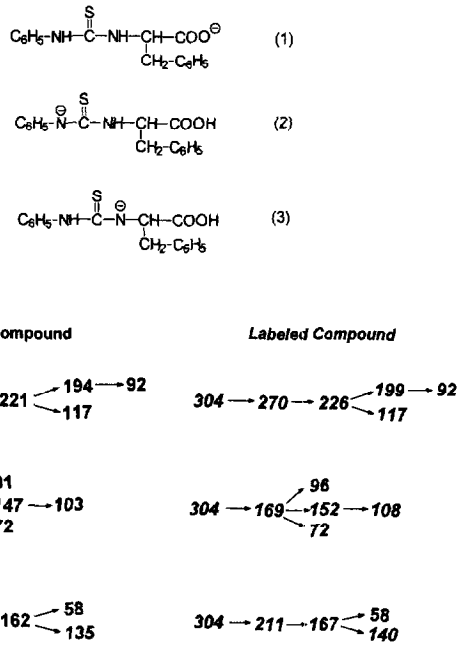


Figure 2. ESI-MS/MS product ion spectrum from the $^2H_5$ labeled PTC-Phe $[M-H]^-$ anion. It shows that peaks at m/z 299, 265, 255, 221, 206, 194, 164, 162, 147, 135, and 103 in Figure 1 were shifted by five mass units higher in this spectrum.

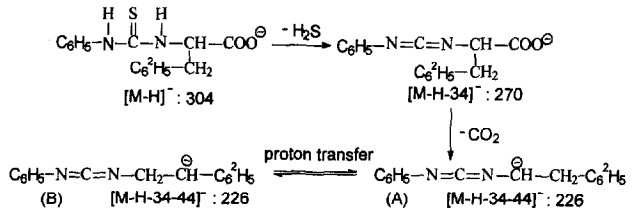


Scheme II. Three ionizable acidic sites in the PTC-Phe molecule and the observed consecutive steps from the three ionization sites of PTC-Phe $[M-H]^-$ ion. Shifted fragments and common ions can be visualized in Schemes III–V.

It should be noted that a similar fragmentation is observed from PTC-tyrosine (data not shown). The fourth ionization site in this molecule does not induce obvious new fragmentation. Since the spectrum of PTC-Phe is one of those showing the most abundant fragmentations, the discussion was focused on this compound. To understand the fragmentation mechanism, several tandem mass spectrometry experiments were performed on the fragments present in the ESI spectrum.

Three deprotonated acidic sites in the molecule are observed as shown in Scheme II. The most obvious is located on the carboxylate group (1). Deprotonation can also occur on both the NH groups (2) and (3), the one on the aniline side being the most acidic. In the product ion spectrum, the fragments that appear at m/z 265, 221, and 164 are very intense peaks, while the one at m/z 206 is relatively weak. We will show that the three fragmentation pathways originate from the three ionization sites and that the order of intensities just mentioned reflects roughly the acidities. The observed consecutive steps are also displayed in Scheme II, which shows that there are obviously three different fragmentation pathways [(I)–(III)].

The formation of the shifted fragment ions and of the common ions can be visualized in Schemes III–V below, which show all the possible fragmentations of this molecule from these three ionization sites. Some inter-



Scheme III(a). Major fragmentations of $[M-H]^-$ produced by deprotonation from the carboxylate site. Stabilized conjugate anion (A) and its tautomeric benzylic anion (B).

mediates in these reaction pathways are indicated as anion/neutral complexes to tentatively rationalize these fragmentation processes.

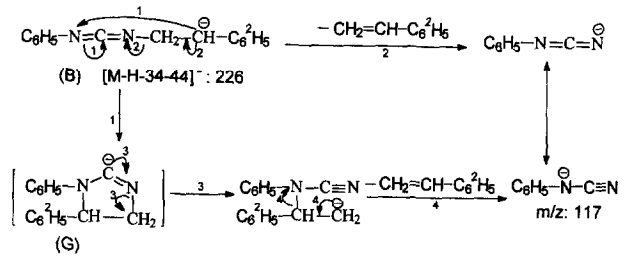
Deprotonation of PTC-Phe on Carboxylate Group (1)

Starting from structure (1) of the deuterated carboxylate anion, $C_6H_5NHC{=}SNHCH(CH_2C_6{}^2H_5)COO^-$, one can explain the peak at m/z 270, corresponding to the loss of H_2S. Figure 3 illustrates the CID spectrum of this labeled fragment anion at m/z 270. It shows the loss of CO_2 as a base peak, yielding a stabilized conjugated anion $C_6H_5{-}N{=}C{=}N{-}CH^-{-}CH_2{-}C_6H_5$ [(A), m/z 226, $[M\text{-}H\text{-}H_2S\text{-}CO_2]^-$]. Consecutive fragmentations occur through either the anions $C_6H_5{-}N{=}C{=}N{-}CH^-{-}CH_2{-}C_6{}^2H_5$ [(A), m/z 226] or its tautomer benzylic anion $C_6H_5{-}N{=}C{=}N{-}CH_2{-}CH^-{-}C_6{}^2H_5$ [(B), m/z 226] formed by proton transfer due to the stability resulting from conjugation. It is shown in Scheme III(a).

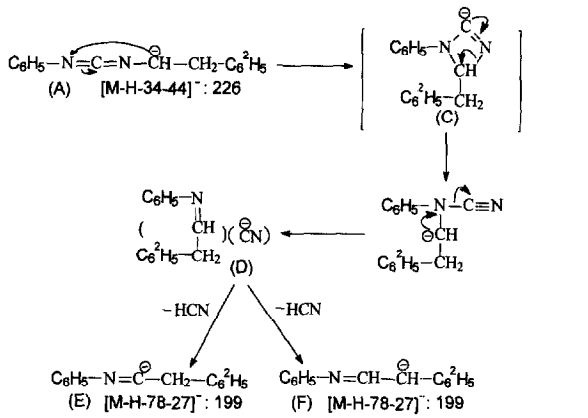
A common ion at m/z 117 displayed in Figures 1, 2, and 3 can be rationalized by the fragmentation of (B) shown in Scheme III(b).

A competing loss of 27 u (HCN) is observed from the fragment at m/z 226, yielding the fragment at m/z 199 observed in Figure 3. The product ion spectrum of the anion at m/z 226 shows this loss of 27 Da (spectrum not shown), which confirms the origin of this fragmentation. This loss of HCN is not straightforward to interpret. It can be explained by two fragmentation pathways displayed in Scheme III(c) and III(d), starting from either ion (A) or (B).

These reactions may occur with anchimeric assistance of the C atom to give the four- and five-membered ring species (C) and (G) shown in Scheme III(c) and III(d). The cyclic transition states consecutively may



Scheme III(b). A common ion at m/z 117 (Figures 1–3) can be rationalized by the fragmentations of (B).

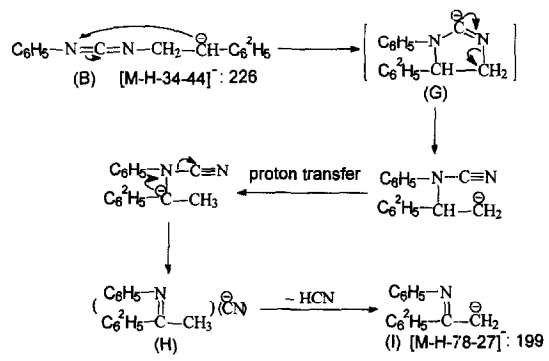


Scheme III(c). Loss of HCN can be explained by the fragmentation of (A).

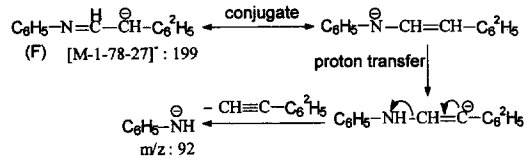
yield two cyanide ion complexes (D) and (H) by the displayed rearrangements. From (D) there are two possible alternative fragmentation pathways that lead to two competing losses of HCN. These reactions produce two tautomeric anions (E) and (F) at m/z 199. Similarly, the complex ion (H) leads also to a loss of HCN, which produces the anion (I) at m/z 199. The reactions for the losses of HCN are not simple cleavages as shown in Scheme III(c) and III(d). They involve a hydrogen transfer and some cleavages and formations of bonds, a four- or five-member cyclic transition, and a cyanide ion complex, finally leading to the eliminations of HCN with the formations of several stabilized anions.

The bound cyanide anion of ion complex (D) deprotonates both neutral portions at the underlined positions: $C_6H_5{-}N{=}\underline{CH}{-}CH_2{-}C_6{}^2H_5$ and $C_6H_5{-}N{=}CH{-}\underline{CH_2}{-}C_6{}^2H_5$, forming an $C_6H_5{-}N{=}C^-{-}CH_2{-}C_6{}^2H_5$ anion [(E)] and $C_6H_5{-}N{=}CH{-}C^-H{-}C_6{}^2H_5$ anion [(F)].

Anion (F) also may form $C_6H_5N^-H$ at m/z 92 by loss of $C_6{}^2H_5C{\equiv}CH$ through the proton transfer shown in Scheme III(e). The fragment ion at m/z 92 is observed in the product ion spectra from m/z 226 and 199 (spectra not shown). The structure of anion at m/z 92 was confirmed by selecting this anion as a precursor. The expected reaction, the elimination of CNH (not HCN) to form the cyclopentadienyl anion $C_5H_5^-$, was observed



Scheme III(d). Loss of HCN can be explained by the fragmentation of (B).



Scheme III(e). (F) can form an anion at m/z 92 by loss of $C_6{}^2H_5$–C≡CH through proton transfer.

(spectrum not shown). This reaction corresponds to a known fragmentation of deprotonated aniline ($C_6H_5N^-H$, m/z 92) [12].

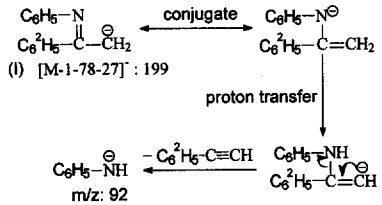
The cyanide anion in the ion/molecule complex (H) can deprotonate the methyl group to form (I) $C_6H_5N{=}C(C_6{}^2H_5)CH_2^-$ (m/z 199), which consecutively forms the fragment anion at m/z 92 by loss of $C_6{}^2H_5C{\equiv}CH$ through the proton transfer shown in Scheme III(f).

The fragmentations in the spectrum displayed in Figure 3 can be rationalized as proceeding through the cyanide ion complexes (D) and (H). There is no obvious fragmentation that allows to distinguish between these two fragmentation pathways.

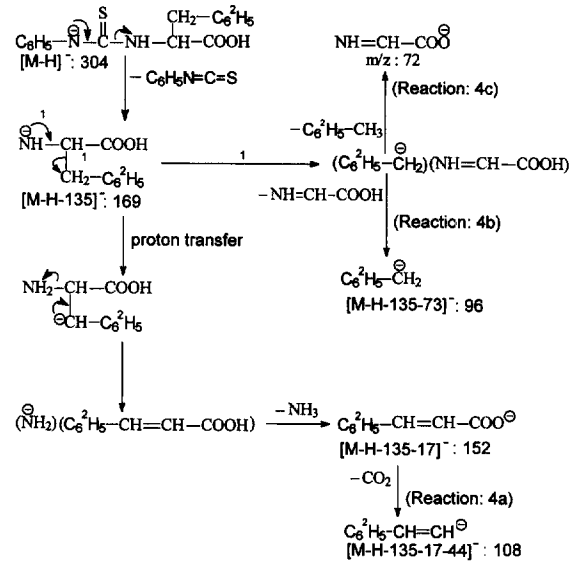
It is worth noting a weak anion at m/z 255 in Figure 1 and m/z 260 in Figure 2 that corresponds to $[M\text{-}H\text{-}CO_2]^-$. Such an anion is unstable by the absence of conjugation. All these observed reactions do not involve loss of hydrogen from the phenyl rings.

Deprotonation of PTC–Phe on Both NH Groups

$C_6H_5N^-C{=}SNHCH(CH_2C_6{}^2H_5)COOH$ *anion* (2). Some important fragments cannot be explained through the carboxylate anion, but by deprotonation from the thioamide groups. We start first from the deprotonation from the thioanilide hydrogen. The characteristic fragmentation of this anion is the observed loss of $C_6H_5N{=}C{=}S$ (135 u), yielding an anion at m/z 164 in Figure 1 and m/z 169 in Figure 2, respectively. This anion has the structure of the deprotonated amino acid, ^-NH–$CH(CH_2$–$C_6{}^2H_5)$–COOH. By selecting this labeled anion at m/z 169 as a precursor in the ion source (Figure 4), the observed spectrum can be explained by the reaction mechanisms shown in Scheme IV. This same fragmentation is observed from the deprotonated molecular ion of pure $^2H_5$-phenylalanine (spectrum not shown). The origin of the fragments, proven by the deuterium labeling, are obvious from the reactions shown in Scheme IV.

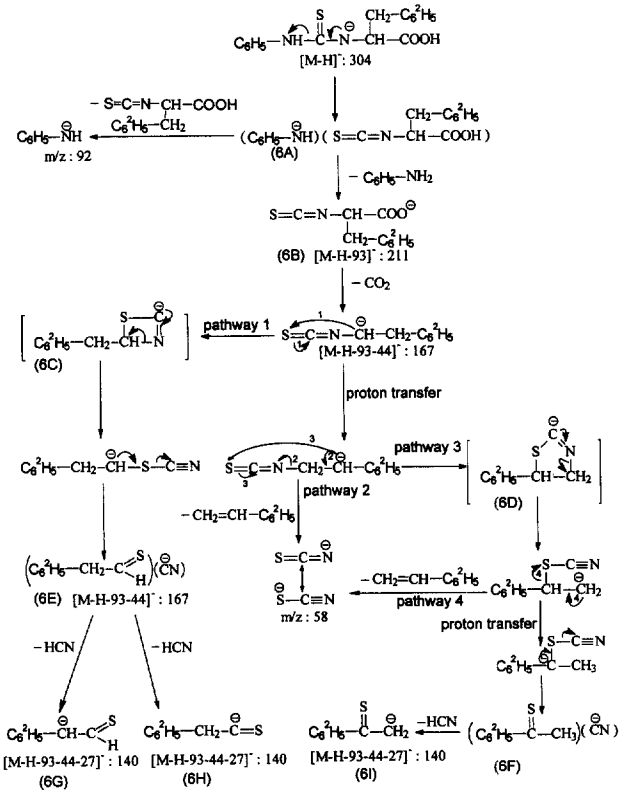


Scheme III(f). (I) can form an anion at m/z 92 by loss of $C_6{}^2H_5$–C≡CH through proton transfer.



Scheme IV. Fragmentations of $[M\text{-}H]^-$ produced by deprotonation from the thioanilide site.

The base peak at m/z 152 results from the loss of NH_3. Subsequent loss of CO_2 yields the fragment at m/z 108. This can be best explained by a proton transfer, yielding the benzylic-type anion NH_2–$CH(COOH)$–$CH^-C_6{}^2H_5$. Through the formation of an ion–molecule complex $(^-NH_2)(C_6{}^2H_5$–CH=CH–COOH),



Scheme V. Fragmentations of $[M\text{-}H]^-$ produced by deprotonation from the thioamide site.

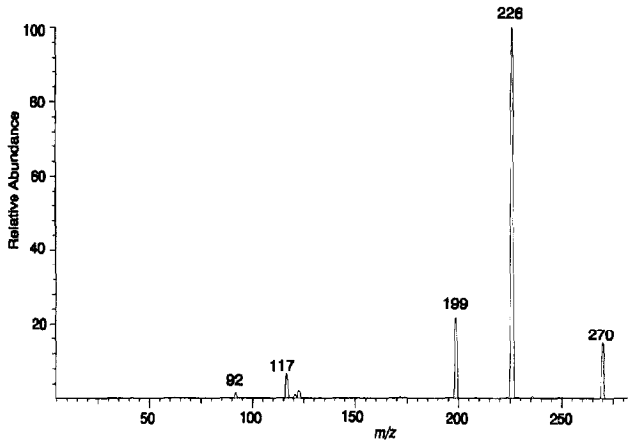


Figure 3. ESI-MS/MS product ion spectrum from the mass-to-charge ratio at 270, resulting from the loss of H_2S from the $[M-H]^-$ anion. This spectrum can be rationalized as shown in Schemes III(a)–III(f).

the loss of NH_3 and the sequential loss of CO_2 produce the stabilized conjugate anion $C_6{}^2H_5$–CH=CH^- at m/z 108. It can be rationalized in reaction (4a) in Scheme IV. Alternatively, another pathway results from reactions (4b) and (4c) shown in Scheme IV, characterized by a complex containing the benzyl anion ($C_6{}^2H_5$–$CH_2{}^-$)(NH=CH–COOH). Either a direct displacement yields a stabilized deuterated benzyl anion $C_6{}^2H_5CH_2{}^-$ (m/z 96) or the deuterated benzyl anion deprotonates NH=CH–COOH to yield the anion NH=CH–COO^- at m/z 72 by loss of $C_6{}^2H_5$–CH_3. Thus, all the fragments observed can be explained.

It is worth noting that a common neutral loss of 135 u, which corresponds to the loss of C_6H_5N=C=S, is observed from all the product ion spectra of the deprotonated PTC amino acids and dipeptides in a mixture (product ion spectra not shown). The six amino acids and three dipeptides PTC derivatives of this mixture, obtained by the same procedure as described above, can be detected selectively by the neutral loss

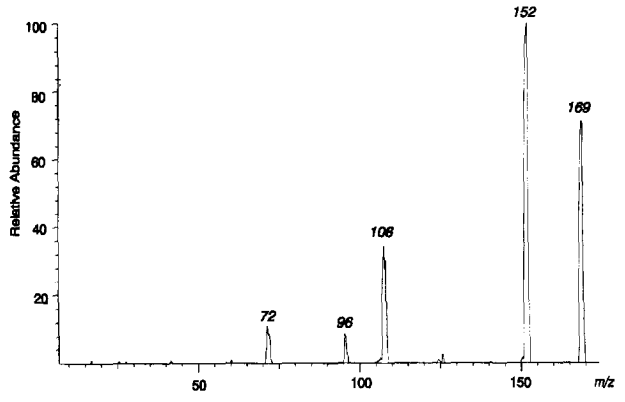


Figure 4. ESI-MS/MS product ion spectrum from the deprotonated amino acid, resulting from the loss of the derivatization reagent from the $[M-H]^-$ anion. This spectrum can be rationalized as shown in Scheme IV.

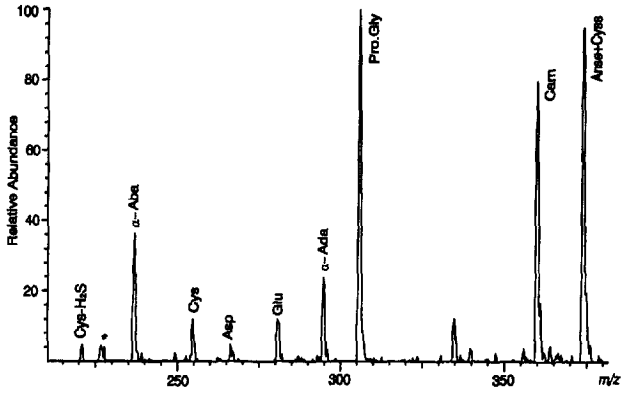


Figure 5. Neutral loss spectrum of 135 Da from a mixture of six amino acids and three dipeptides PTC derivatives. This spectrum show the general trend concerning the common fragmentation of PTC amino acid derivatives. α-Aba: α-amino-butyric acid; Cys: cysteine; Asp: aspartic acid; Glu: glutamic acid; α-Ada: α-amino-adipic acid; Pro.Gly: proline.glycine; Carn: carnosine; Anse: anserine; Cyss: cystine. A byproduct (thiocarbanilide) is marked by an asterisk.

scan function of 135 u (Figure 5). This common fragmentation pathway shows the general trend of our work, which is to develop a more efficient method to detect amino acids in biological fluids using the classical derivative formation.

C_6H_5NHC=$SN^-CH(CH_2C_6{}^2H_5)COOH$ anion (3). The reactions which correspond to this anion are summarized in Scheme V. Symbols (6A)–(6I) represent the different species shown in Scheme V. The cleavages and formations of bonds produce an ion complex (6A) ($C_6H_5NH^-$)(S=C=N–CH(COOH)$CH_2C_6{}^2H_5$) first. Direct displacement yields C_6H_5–NH^- at m/z 92, the same structure mentioned in Scheme III(e) and III(f). Reactions of C_6H_5–NHC=SN^-CH(COOH)($CH_2C_6{}^2H_5$) through the ion complex (6A) account for all other fragmentations. Direct cleavage yields the expected product (6B) at m/z 211 by loss of aniline $C_6H_5NH_2$ (Figure 2). By selecting this labeled anion at m/z 211 as a precursor in the source, one obtains the spectrum displayed in Figure 6. The base peak at m/z 167 corresponds to the loss of CO_2. The formation of the stabilized conjugate S=C=N–$^-$CH–CH_2–$C_6{}^2H_5$ (m/z 167) then yields several possible tautomeric structures of a fragment at m/z 140 by loss of 27 u. These eliminations of HCN (27 u) are similar to those displayed in Scheme III(c) and III(d). Pathway 1 in Scheme V may occur with anchimeric assistance of the C atom to give the four-membered ring transition species (6C), and then through rearrangements of the bonds, can finally be rationalized as proceeding through the cyanide ion complex (6E). Losses of HCN result from (6E), yielding two possible anions (6G) and (6H) at m/z 140. Another possible pathway after the loss of CO_2 is through a specific proton transfer from the hydrogen of the benzylic position to the position underlined, S=C=N–$^-$CH–CH_2–$C_6{}^2H_5$, forming a stabilized ben-

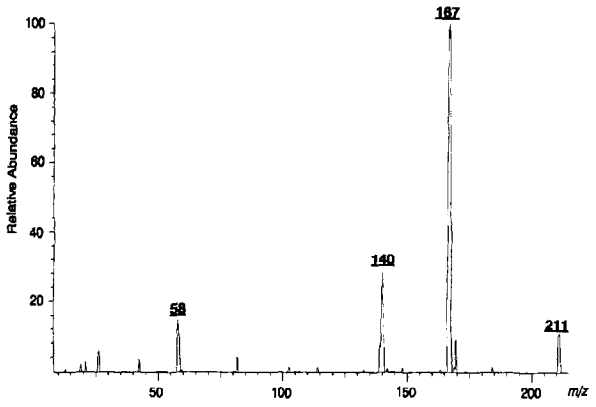


Figure 6. ESI-MS/MS product ion spectrum from the mass-to-charge ratio at 211, resulting from the loss of aniline from the $[M-H]^-$ anion. This spectrum can be rationalized as shown in Scheme V.

zyl-type anion $S{=}C{=}N{-}CH_2{-}^-CH{-}C_6{}^2H_5$. This one forms $S{=}C{=}N^-$ anion at m/z 58 by direct loss of $CH_2{=}CH{-}C_6{}^2H_5$ as shown in pathway 2, Scheme V. This $^-S{-}C{\equiv}N$ can also be formed by loss of $CH_2{=}CHC_6{}^2H_5$ through a five-membered ring transition (6D) and the reactions shown in pathway 4, Scheme V. The loss of HCN may also occur in a similar fashion through (6C), with anchimeric assistance of the C atom to give the five-membered ring transition state (6D). Rearrangements of the bonds and finally the formation of the cyanide ion complex (6F) may explain this loss of HCN. The cyanide anion can deprotonate the thioketone methyl group to yield (6I) at m/z 140.

Conclusion

We have observed several series of reactions from the same deprotonated molecule that differ by the location of the negative charge site. There are three main independent fragmentation pathways, which suggest that they could originate from the three different deprotonation sites.

The reaction pathways originating from these three deprotonation sites indeed allow to rationalize the observed fragmentations. Conjugation always lowers the energy of an unsaturated system by allowing the π electrons to be delocalized. This behavior is just what explains the proposed rearrangements and fragmentations.

This study shows that one of the main fragments, resulting from the loss of the derivatization reagent (135 Da), is the anion of the deprotonated free amino acid. Preliminary results have shown that this also occurs for other amino acids. Thus, this neutral loss of 135 Da could be used to selectively detect PTC amino acid derivatives in mixtures. The product ion from these $[M-H-135]^-$ ions, as shown here for the phenylalanine, yields information on the structure of the amino acid. This study shows that it could be applied to even trace amounts.

Acknowledgments

The authors are grateful to R. Rosenberg and A. Spote for their skilled technical assistance and to Dr. N. Viseux and Dr. V. Stroobant for their kind help. The authors are indebted to the Belgium National Research Fund (FNRS) for financial support.

References

1. Vetter, W. In *Biochemical Applications of Mass Spectrometry*; Waller, G. R.; Dermer, O. C., Eds.; Wiley: New York, 1980; pp 439–461.
2. Millington, D. S.; Kodo, N.; Naoto, T.; Diane, R.; Chace, D. H. *Int. J. Mass Spectrom. Ion Processes* **1991,** *111,* 211–228.
3. Rashed, M. S.; Ozand, P. T.; Bucknall, M. P.; Little, D. *Pediatr. Res.* **1995,** *38,* 324–331.
4. Chace, D. H.; Hillman, S. L.; Millington, D. S.; Kahler, S. G.; Adam, B. W.; Levy, H. L. *Clin. Chem.* **1996,** *42,* 349–55.
5. Chace, D. H.; Hillman, S. L.; Millington, D. S.; Kahler, S. G.; Roe, C. R.; Naylor, E. W. *Clin. Chem.* **1995,** *41,* 62–68.
6. Chace, D. H.; Millington, D. S.; Terada, N.; Kahler, S. G.; Roe, C. R.; Hofman, L. F. *Clin. Chem.* **1993,** *39,* 66–71.
7. Feste, A. S. *J. Chromatogr.* **1992,** *574,* 23–34.
8. Pramanik, B. C.; Moomaw, C. R.; Evans, C. T.; Cohen, S. A.; Slaughter, C. A. *Anal. Biochem.* **1989,** *176,* 269–277.
9. Pramanik, B. C.; Hiton, S. T.; Millington, D. S.; Doureville, T. A.; Slaughter, C. A. *Anal. Biochem.* **1988,** *175,* 305–318.
10. Ersser, R. S.; Davey, J. F. *Med. Lab. Sci.* **1991,** *48,* 59–71.
11. Chait, B. T.; Wang, R.; Beavis, R. C.; Kent, S. B. H. *Science* **1993,** *262,* 89–92.
12. Raftery, M. J.; Bowie, J. H. *Aust. J. Chem.* **1988,** *41,* 1477–1489.